

Transcriptional interference between the EBV transcription factors EB1 and R: both DNA-binding and activation domains of EB1 are required

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ABSTRACT

The switch from latency to a productive infection in EBV-infected B cells is linked to the expression of two viral sequence-specific DNA-binding transcription factors called EB1 and R. EB1 shares sequence homologies with the bZIP family of proteins in the basic region required for specific DNA interaction. Here, we provide evidence that EB1 and R can synergistically activate specific transcription, and that overexpressed, unbound EB1, represses the R-induced transcription ('squelching'). In order to identify the EB1 domains involved in transcriptional activation, transcriptional synergy and transcriptional repression, we performed extensive mutagenesis of the EB1 protein. Results show that five segments (region 1 to region 5), localized at the N-terminus of EB1 exhibit characteristics of activating domains, since they are required for full transcriptional activity, without obvious role in DNA-binding, or the nuclear localization. Two domains rich in basic amino-acids are required for the nuclear localization of EB1. One domain is within the basic region B, also necessary for specific and stable interaction between EB1 and its cognate DNA sequences. It is also shown that the 'activation' domain, and more surprisingly the DNA-binding domain of EB1, may interact with a factor(s), essential for R-induced activation, and probably required for synergy between EB1 and R.

INTRODUCTION

The Epstein–Barr virus (EBV) is a human herpes virus which infects and immortalizes peripheral B lymphocytes, resulting in the establishment of a latent infection. In such latently infected B cells, the entire EBV genome is maintained largely as an extrachromosomal circular DNA molecule, and viral expression is restricted to a few genes (1). In some B cell lines, between 0.5% and 5% of the cells produce virus, and their number can be increased by various chemical agents including the tumor promoter 12-O-tetradecanoyl-phorbol 13-acetate (TPA) (2). The TPA-induced production of virions seems to be linked to the synthesis of the EBV transcription factor EB1 (3; 4; 5; 6), which is encoded by the BZLF1 open reading frame (ORF) (Figure 1A). EB1 is expressed from two promoters, PZ and PR , either as a monocistronic mRNA of 1kb, or as 3Kb and 4 kb long mRNA generated by alternative splicing. The latter expresses both EB1 and the enhancer factor R (Figure 1A) (7). The PZ promoter responds to TPA, and induction is mediated by an AP-1 site identical to and present in a similar location to one in the c-jun promoter (Figure 1A)(8; 9; 10). Once made, EB1 positively autoregulates promoter PZ through two binding sites (9; 11), and activates the promoter PR controlling the expression of the bicistronic mRNA (Figure 1A)(12; 13; 14; 15). Then, EB1 and R activate the EBV early promoters and the origins of replication, ORIlyt, which are active only during the lytic cycle of the virus (16).

EB1 was originally described (17), as being related to the bZIP family of proteins (Figure 1B)(18). Significant homology is observed in the domain rich in basic amino acids that is thought to directly contact the DNA in this class of proteins (19). This domain has been proposed to consist of two clusters, basic region A (BR-A) and basic region B (BR-B), separated by a spacer of alanines (Figure 1B)(18). These clusters are similar among all the bZIP proteins and are also conserved in EB1 (Figure 1B). However, the stable interaction of the bZIP proteins with their cognate DNA sequences occurs only when a dimerization domain, a heptad repeat of leucines located immediately C-terminal to the basic domain, is functional (Figure 1B) (19; 20). The EB1 dimerization domain is not a repeat of leucines (Figure 1B), but conforms to the more general requirement for the formation of a coiled coil with the presence of the 4–3 repeat of hydrophobic residues (Figure 1B) (21; 22; 23; 24). Therefore, the DNA-binding domain of EB1 (basic plus dimerization regions), is likely to be restricted to the C-terminal part of the protein, while the N-terminal part of the protein may contain the

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transcriptional 'activating' domain (25). However, the precise location of the domains required for transcriptional activation, have not been mapped by detailed mutagenesis of the EB1 protein. Likewise, the nuclear localization and the stability of mutant proteins have not been examined.

Here, it is reported that R and EB1 can activate alone or synergistically specific transcription, and that unbound EB1 inhibits the R-induced activation in a dose-dependent manner. In order to precisely locate the protein domains required for transcriptional activation, synergism, and repression, we generated several deletion, substitution and domain exchange mutants in the EB1 protein. Five N-terminally located segments of EB1 (region 1 to region 5, Figure 3A) are required for full transcriptional activation, and display properties of 'activation' domains, since they are not required for DNA-binding and nuclear localization. The basic region B of EB1 contains one of the two sequences required for the nuclear localization of the protein, and directs both specific and stable DNA-binding. Using 'domain-swapping' and several other mutants, we also found that region 2 in the activation domain and the basic region B are required for the interaction between EB1 and a factor (s) necessary for R-dependent activation of transcription, and for synergy between EB1 and R.

MATERIALS AND METHODS

Recombinant plasmid constructions

The construction of plasmids pM and pM19 has been described elsewhere (9). Plasmid pβG was constructed by ligating the double strandedoligonucleotide 5'-CTGCAGCTCGCCTT-CTTTTATCCTCTTTTGTCGACC-3', 5'-TCGAGGTCGAC-AAAAGGATAAAAGAAGGCGAGCTGCAG-3' containing the EBV DR/DL promoter TATA box (map positions 52801 to 52826 on the EBV B95-8 sequence; 26), into plasmid pG2 cut by XhoI and PvuII. Plasmid pβGR was made by inserting the EBV DR/DL R-responsive enhancer (map positions 53523 to 53593 on the EBV B95-8 sequence; 26) into the SacI site of plasmid pβG. Plasmid pG221 has been made by ligating the DR promoter proximal sequences (13) into plasmid pG2, and contains four EB1 binding sites: TGTGCAA (ZRED1), TGAGCAA (ZRED2), TGTGTGA (ZRED3) and TGTGTAA (ZRED4) (Figure 4A). Each insertion mutant was sequenced before use. The construction of the EB1 expression vector pKSVZ41 and the R expression vector pKSVR have been described elsewhere (7). Plasmid pSV2β expresses a chimeric SV40-β-globin RNA and was cotransfected as an internal control for transient expression experiments (14).

Construction of EB1 mutants

Plasmid pKSVZ41 containing a cDNA coding for EB1 (7) was cut with restriction enzymes AluI, HaeIII, HincII SmaI and PvuII, in the presence of ethidium bromide so as to produce a maximum of single cut linear molecules (27). Linear DNA was isolated after agarose gel electrophoresis and ligated directly to 12-bp Bgl II linker oligonucleotides. The insertion location and the number of linkers inserted was determined by sequencing. When necessary, plasmids were extensively digested with Bgl II, religated and sequenced to ensure that only one linker was inserted. Deletion mutants were generated by cutting different insertion mutants with Bgl II, and religating the appropriate DNA fragments to generate in phase internal deletions. Single or multiple amino-acid changes in mutants Z306, Z310 and Z311, as well as exchange of protein domains in mutants ZJ, ZJA and ZJB were performed by using the MUTA-GENE M13 in vitro mutagenesis kit from BIO-RAD. All the mutants produced were sequenced before use.

Production of EB1 mutant proteins in vitro

Mutated EB1 cDNAs were subcloned in plasmids pSPT18 or pSPT19 (Boehringer Mannheim). Cloned inserts within the polylinker region were transcribed from either the SP6 or the T7 promoters. The RNA obtained were used to program protein synthesis in messenger-dependent rabbit reticulocyte lysates (Promega) using ^{14}C-L-Leucine or ^{35}S-L-Methionine.

Transfection procedure and RNA analysis

HeLa cells were grown in DMEM medium supplemented with 10% FCS, and seeded at 10^6 cells per 100 mm Petri dish 4h before transfection. The cells were transfected by the $CaPO_4$-DNA precipitate method (28). Usually, 15 μg of DNA was added which included: 1μg of plasmid pSV2β as an internal control for transfection, different amounts of EB1 or R expression vectors and pSV0 DNA when required to keep the amount of SV40 early promoter sequences constant, plus pUC18 DNA up to the 15μg. Cytoplasmic RNAs were extracted as described (14). Total cytoplasmic RNA (10 to 40μg) was hybridized overnight at 30°C in 50% formamide, 0.3 M Na Cl, 0.01M Tris-HCl, pH 7.4, to 5'-^{32}P-labelled synthetic single-stranded DNA probes. The hybrids were digested for 2h at 20°C with 50 U of S1 nuclease per 20 μg of RNA. The size of the S1-protected DNA fragments was analysed on 8% (w/v) polyacrylamide-8.3 M urea gels.

Electrophoretic Mobility Shift Assay (EMSA)

2 μl of in vitro translation extracts were incubated with 1.5×10^5 cpm of ^{32}P-labelled-double-stranded oligonucleotides containing different DNA-binding sites. Incubations were carried out at either 20°C or 4°C for 30 minutes, in 1mM $MgCl_2$, 20mM HEPES-KOH (pH 7.9), 0.5 mM DTT, 0.5 mM PMSF, 140mM KCL, 20% glycerol, 1μg poly dI:dC. The mixture was loaded onto a 4% polyacrylamide 0.25×TBE gel (crosslinked 29 to 1 with bisacrylamide). The DNA-protein complexes were separated from the non-complexed DNA by migration at 10 V/cm either at 20°C or 4°C. The results of the experiments were visualized by autoradiography.

Immunoblots

The 5×10^6 HeLa cells were collected 72h following the transfection, and lysed by Nonidet P-40. Nuclei were separated from cytoplasm by centrifugation. Each subcellular fraction was resuspended in SDS reducing buffer (O.O5M Tris-HCL (pH 6.8), 10% Glycerol, 0.1% SDS, 0.14M β-mercaptoethanol, O.O5% bromophenol blue), and boiled for 2 min. One third of each extract was electrophoresed on SDS-PAGE and transferred to a nitrocellulose filter before incubation with human anti-EB1 antibody.

RESULTS

R and EB1 act synergistically to activate transcription

Transcriptional activation by each or by both of the transcription factors EB1 and R, was investigated by HeLa cells cotransfection of the corresponding expression vectors together with a plasmid carrying the EBV early promoter PM, linked to the mRNA-coding element of the rabbit β-globin gene. Promoter PM contains an EB1-responsive element superimposed with a

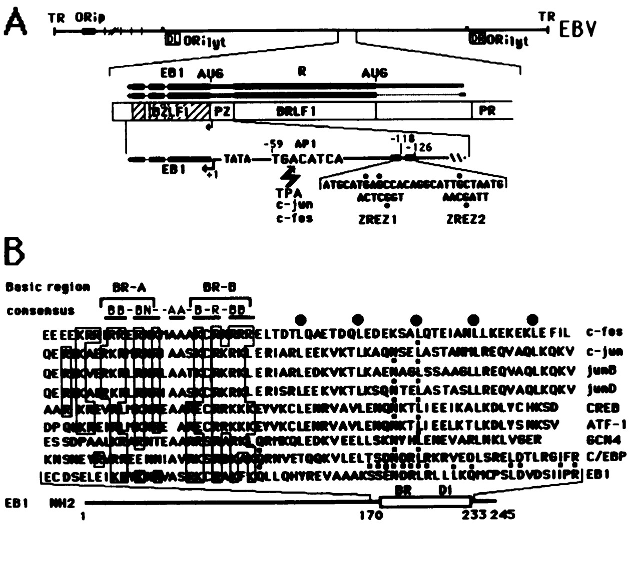

Figure 1. Autoregulation and structure of EB1. **A)** Two EBV open reading frames, BZLF1 and BRLF1, code for the transcription factors EB1 and R respectively. The expression of EB1 from promoter PZ is positively autoregulated by direct binding of EB1 to ZREZ1 and ZREZ2. Promoter PZ contains also an AP-1 site that could mediate TPA/Jun/Fos induction. Promoteur PR directs the expression of bicistronic mRNA coding for both EB1 and R. The ORIlyt are transactivated by EB1 and R. **B)** EB1 is partially homologous to the bZIP family of proteins, in the basic region (BR) (BR-A, alanine spacer AA, BR-B). Basic amino-acids residues are delineated by thin boxes. The EB1 dimerization region (Di), is not a repeat of leucines at every seventh position (black circles), but has some conserved amino-acids residues (black squares), especially with C/EBP.

conserved AP-1 binding site (9) and an R-dependent enhancer (29; 30), where a unique R-binding site has been localized between positions −373 and −390 upstream from the major transcription start (Figure 2A) (Gruffat, personal communication). In HeLa cells, the PM promoter had a weak basal activity (Fig. 2B, lane 1). This basal activity was stimulated 1.9 fold upon transfection with the EB1-expression vector (Fig. 2B, lanes 2). The activity of the PM promoter was stimulated 10 fold upon transfection with the R-expression vector (Figure 2B, lane 3). Interestingly enough, when coexpressed, EB1 and R activated the PM promoter 37 fold (Figure 2B, lane 4). Therefore EB1 and R appeared to act synergistically, since their combined effect (37 fold), was more than additive (11.9 fold), as quantitated by counting the radioactivity in the S1 protected DNA fragments corresponding to specifically initiated RNA. Using a reporter gene composed of three synthetic double stranded oligonucleotides containing the TATA box, the AP-1 site and the R-binding site of the EBV early promoter PM, respectively,we obtained similar results, indicating that synergism requires only these three promoter elements (not shown).

In all the transfections described here, 0.5μg of plasmid DNA pSV2β with the SV40 early promoter directing the expression of an SV40-β-globin chimeric RNA, was included as an internal control. This was also done to evaluate the activity of the SV40 early promoter which is employed for the expression of EB1 and R. In addition, the amount of SV40 early promoter was kept constant in every transfection by adding, when required, plasmid pSV0 containing only the SV40 early promoter sequences. A comparable amount of specifically initiated SV40-β-globin transcripts (labelled SV) was found in each transfection presented, suggesting that the results of S1 nuclease mapping could be compared quantitatively.

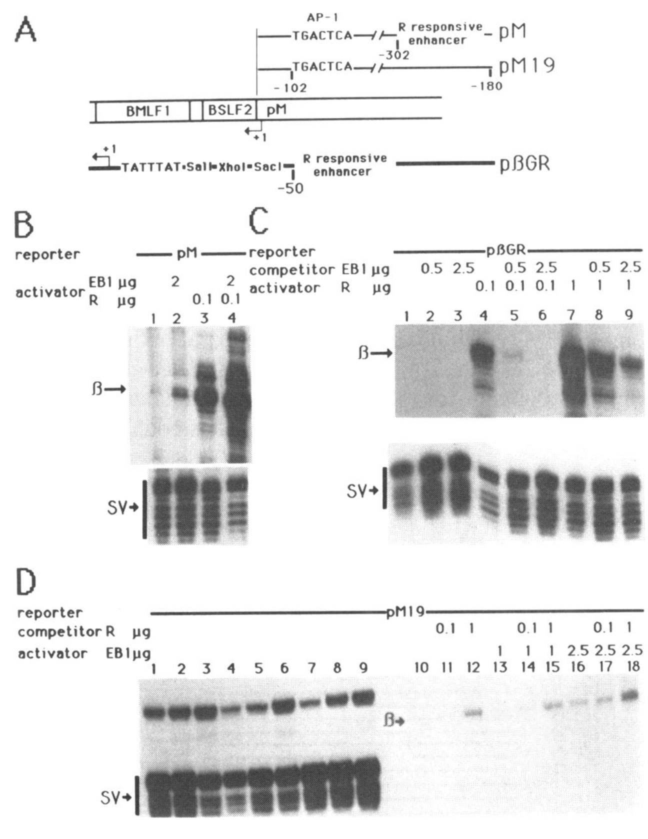

Figure 2. Synergistic activation and transcriptional repression by EB1 in HeLa cells. **A)** Structure of promoters pM, pM19 and pβGR. **B)** S1 nuclease analysis was performed with RNAs extracted from HeLa cells transfected with the reporter gene pM (lanes 1 to 4), either alone (lane 1), or with an EB1 expression vector (lane 2), or with an R expression vector (lane 3), or with both (lane 4). **C)** S1 nuclease analysis of RNAs extracted from HeLa cells transfected with the reporter gene pβGR (lanes 1 to 9), either alone (lane 1), or in the presence of increasing amounts of EB1 (lanes 2 and 3), or in the presence of 0.1μg of R-expressing vector plus increasing amounts of EB1 (lanes 4 to 6), or in the presence of 1 μg of R-expressing vector plus increasing amounts of EB1 (lanes 7 to 9, and lanes 11 to 13). **D)** S1 nuclease analysis of RNAs extracted from HeLa cells transfected with the reporter gene pM19 (lanes 10 to 18), either alone (lane 10), or with increasings amounts of R-expressing vector (lanes 11 and 12), or with 1μg of EB1 and increasing amounts of R-expressing vectors (lanes 13 to 15) or with 2.5 μg of EB1 and increasing amounts of R-expressing vectors (lanes 16 to 18). In all transfections presented, S1 analysis of SV40 early RNAs (SV) expressed from plasmid pSV2β cotransfected as an internal control is presented (lower panel in Figure 2B, 2D and lanes 1 to 9 in figure 2C). β represents specifically initiated β-globin RNAs.

EB1 represses the R-induced activation

EB1 and R can act synergistically, and this effect could reflect either direct interaction between these factors or indirect interaction through an intermediary factor (s). In the latter case, overexpression of unbound EB1 should repress the R-induced activation ('squelching' (31)). To test this possibility, we first constructed a minimum promoter containing a TATA box and two R-binding sites, located 5′ to the rabbit β-globin gene (Figure 2A, pβGR). This promoter had a weak basal activity (Figure 2C, lane 1), and did not detectably respond to increasing amounts of EB1 (Figure 2C, lanes 2 and 3). This promoter was activated by R, and the activation increased with increasing amounts of R (Figure 2C, lanes 4 and 7). As expected, when increasing amounts of EB1 were expressed together with R, EB1 repressed the R-dependent activation. The repression was seen both at low (Figure 2C, lanes 5 and 6) and at high R concentration (Figure

2C, lanes 8 and 9). As monitored by immunoblotting, the amount of EB1 or R proteins increased following transfection of increasing amounts of expression vector (not shown). It should also be noted that transcription from the SV40 early promoter cotransfected as an internal control, was not affected by cotransfection of the competitor expression vector at all concentrations used, indicating that the factor(s) sequestered by EB1 was not required for SV40 early transcription.

We also investigated whether high R concentrations could affect EB1 activation of the promoter PM carrying only the AP1 site and no R-responsive enhancer (Mutant PM19, Figure 2A). Promoter PM19 had a barely detectable basal activity in HeLa cells (Figure 2D, lane 10), but its activity was detectably increased by high amounts of EB1 (Figure 2D, compare lanes 10, 13 and 16). Although no low affinity R-binding sites could be detected on promoter PM19 (not shown), increasing amounts of R weakly increased the basal activity of promoter PM19 (Figure 2D, lanes 11 and 12). However, not only there was no synergy between EB1 and R, but increasing amounts of R did not repress the EB1-induced activation of promoter PM19, either at low EB1 concentration (Figure 2D, lanes 14 and 15) or at high EB1 concentration (Figure 2D, lanes 17 and 18). In conclusion, since repression of R activation was better seen at high EB1 concentrations, and in the absence of an EB1 DNA-binding site, it appears to be related to 'squelching' (31).

Construction of EB1 mutants

To examine the structural features of EB1 directing transcriptional activation, as well as synergy with, and repression of R-induced transcription, deletion, substitution, insertion, and domain exchange mutants were constructed (Figure 3A and 3C). Mutant Z59 has an insertion of four amino-acids EDLP at position 59. Some of the mutant proteins were expressed in vitro in the rabbit reticulocyte lysate, and used to analyse their DNA-binding

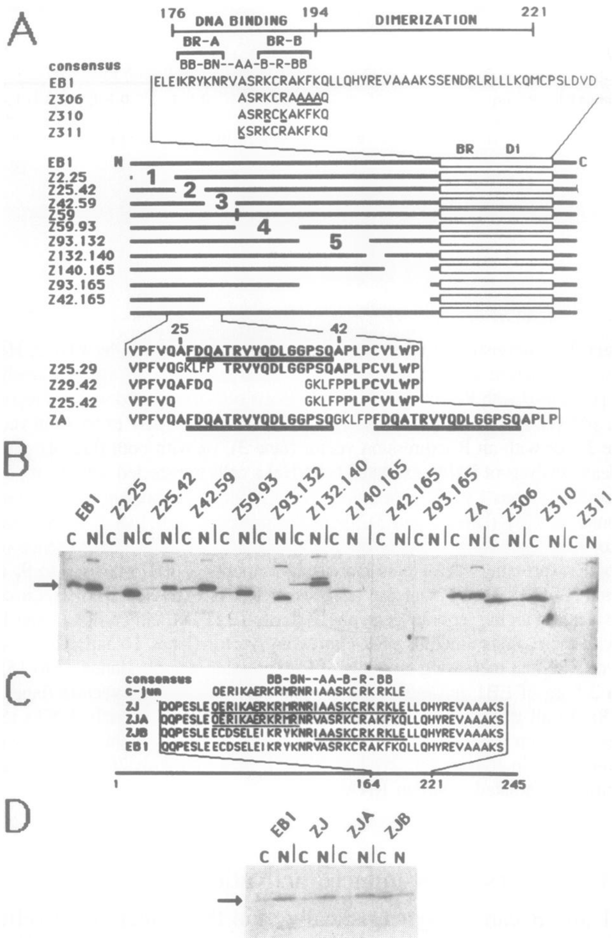

Figure 3. Nuclear localization and stability of EB1 mutants. **A and C)** Schematic representation of EB1 and mutated EB1 variants. The DNA binding region is composed of two clusters of basic amino-acids (B), BR-A and BR-B, separated by a spacer of alanines. The two numbers after Z indicate the extent of amino-acids deletion. Mutants Z306, Z310 and Z311 are substitution-mutants. Mutants ZJ, ZJA and ZJB are domain 'swapping' mutants. Numbers 1, 2, 3, 4 and 5 in mutants Z2.25, Z25.42, Z42.59, Z59.93 and Z93.132 respectively, indicte region 1 to region 5 whose deletion affect the activation potential of EB1 **B and D)** Nuclear localization and stability of EB1 mutants. EB1 mutants were transfected in HeLa cells. Proteins present in the nuclear fraction (N) or in the cytoplasmic fraction (C) were separated on 10% SDS-PAGE, and transferred to nitrocellulose. EB1 protein and mutants were vizualised using an anti-EB1 rabbit antibody. The horizontal arrow indicates where the wild type protein EB1 migrates. Mutants Z42.165 and Z93.165 are indicated by black squares.

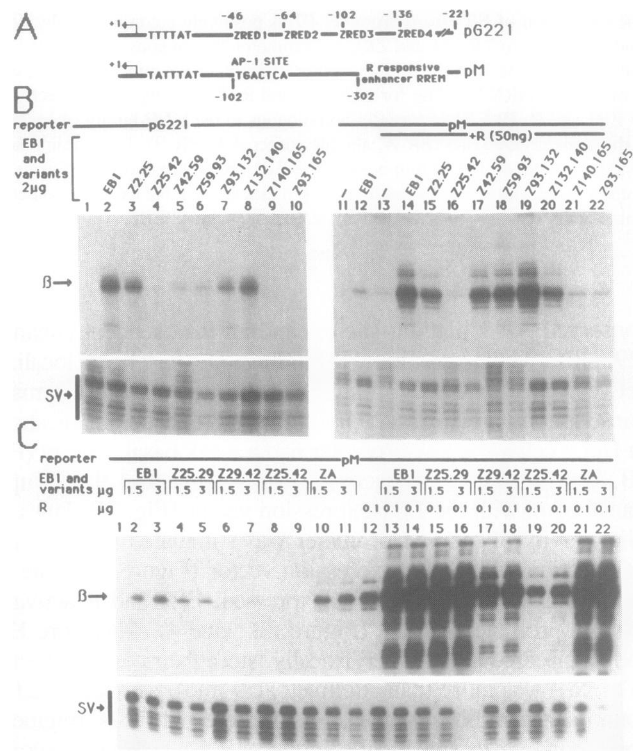

Figure 4. Synergistic activation between EB1 and R requires region 2 in the EB1 activation domain. **A)** Schematic representation of reporter genes pG221 and pM. In the reporter gene pG221, ZRED1 to ZRED4 correspond to the EB1 binding sites described in probes DR1 to DR4 in Figure 6A. **B)** S1 nuclease mapping of RNAs extracted from HeLa cells transfected with the reporter gene pG221 (lanes 1 to 10) or with the reporter gene pM (lanes 11 to 22). The reporter genes were transfected in the absence of any EBV transactivator (lanes 1 and 11), or in the presence 2μg of EB1 (lanes 2 to 12), or in the presence of 2μg of the EB1 deletion mutants presented in the upper part of the panel (lane 3 to 10), of in the presence of 50ng of R (lane 13), or in the presence of 50 ng of R plus 2 μg of the EB1 deletion mutants presented in the upper part of the panel (lanes 14 to 22). **C)** The reporter gene pM was transfected either alone (lane 1), or in the presence of two concentrations of EB1 (lanes 2 and 3), or in the presence oftwo concentrations of the EB1 mutants presented in the upper part of the panel (lanes 2 to 11), or in the presence of R alone (lane 12), or in the presence of R plus two concentrations of EB1 or EB1 mutants (lanes 13 to 22). The genetic content of the EB1 mutants is shown in Figure 3. RNAs expressed from plasmid pSV2β (SV) cotransfected as an internal control, are presented in each panel. β represents β-globin specific transcripts.

activity. Proteins were labelled with ^{14}C-leucine or ^{35}S-methionine and visualised by SDS-PAGE and autoradiography. Equimolar amounts of proteins were incubated at 20°C or 4°C with an excess of ^{32}P-radiolabelled DNA oligonucleotides, to evaluate the relative affinity of the mutants for the different DNA binding sites in vitro. The transactivation capacity of mutated proteins, alone or in synergy with R, as well as the inhibitory effect of these proteins on the R-dependent transcriptional activation were analysed by transient expression in HeLa cells.

First, the subcellular localization of EB1 and of mutated EB1 proteins was determined by SDS-PAGE and immunoblotting, after transfection in HeLa cells, and separation of the nuclear fraction from the cytoplasmic fraction (Figure 3B and 3D). The wild type protein EB1 was found predominantly in the nuclear fraction. Ten mutants, Z2.25, Z25.42, Z42.59, Z59.93, Z93.132, Z132.140,ZA, Z311, ZJ and ZJA were found in majority in the nuclear fraction. The western blot also shows that a comparable amount of the proteins localized in the nucleus was detected by the antibody, indicating that EB1 mutants have comparable stability in HeLa cells. On the contrary, mutants Z140.165, Z93.165, Z42.165, Z306, Z310 and ZJB were found mostly in the cytoplasmic fraction. These results were confirmed by indirect immunofluorescence after transfection in Cos-7 and Hela cells of the relevant mutants (not shown). These results also suggest that EB1 has two sequences required for the nuclear localization. One contains the sequence RKCRAKFK which overlaps with basic region B, while the second one, located between amino-acid 140 and 165, encompasses the sequence RRTRKP.

Region 2 in the EB1 'activation' domain is essential for synergistic activity with the enhancer factor R

We next tested the ability of various deletion mutants localized in the nucleus, to activate transcription from the EBV early promoter DR (Figure 4A, plasmid pG221). Two mutants localized in the cytoplasm, Z140.165 and Z93.165 (Figure 3A), were also used as negative controls. As expected, these mutants, gave negative results in transcriptional activity as compared with EB1 (Figure 4B, lanes 9 and 10). The variants localized in the nucleus allowed us to identify in the N-terminal region of EB1, a set of segments that contributed differently to the EB1 transcriptional activation. These segments have been arbitrarily defined region 1 to region 5 (Figure 3A). As compared to EB1 (lane 2), mutant Z2.25 had a reduced activation potential and defined region 1 (lane 3). Mutant Z25.42 was almost incompetent in the transactivation assay and defined region 2 (lane 4). Mutants Z42.59 and Z59.93 had intermediate activation competence (lanes 5 and 6) as compared to EB1 (lane 2) and Z25.42 (lane 4) and defined region 3 and region 4, respectively. Finally, mutant Z93.132 had about the same activation competence (lane 7) as mutant Z2.25 (lane 3) and defined region 5. None of the deletions presented above modified the DNA-binding properties of EB1 (not shown). The same mutants were analysed for their capacity to activate the PM promoter, in synergy with the EBV enhancer factor R. As shown in figure 4B, promoter PM had a weak basal activity (lane 11), that was increased by EB1 (lane 12) and by R (lane 13). Among the variants that were localized in the nuclei, only mutant Z25.42 (region 2), had no synergistic effect with R (lane 16), whereas variants in which regions 1, 3, 4 or 5 were deleted (lanes 15, 17, 18 and 19 respectively), could still act in synergy with R.

In an attempt to further study the region encompassing amino-acids 25 to 42, we generated mutants Z25.29 and Z29.42 (Figure 3A). Their activity alone and together with R was compared with EB1 and with mutant Z25.42. Mutants Z25.29 and Z29.42 localized in the nucleus, and were stably expressed at a level comparable to wild type EB1 (not shown). We also used higher amounts of EB1 mutants and R proteins in an attempt to vizualized a residual activation potential of the mutants. Deletion of amino-acids 25 to 29 reduced the activity of the mutated protein (Figure 4C, lanes 4 and 5), but did not alter its synergistic effect with R (Figure 4C, lanes 15 and 16), as compared to that observed with R plus EB1 (Figure 4C, lanes 13 and 14). Deletion of amino-acids 29 to 42 decreased further the activity of the mutated protein (Figure 4C, lanes 6 and 7), but again did not impair its synergistic effect with R (Figure 4C, lanes 17 and 18). As already shown, complete inactivation (Figure 4C, lanes 8 and 9) and loss of synergy (Figure 4C, lanes 19 and 20) were only observed when amino-acids 25 to 42 were deleted. Two copies of region 25.42 gave ZA a higher transactivation potential (Figure 4C, lanes 10 and 11) as compared to EB1 (Figure 4C, lane 2 and 3), and synergism between ZA and R was still detected (Figure 4C, lanes 21 and 22). In conclusion, an EB1 region essential for the activation and for synergy with R, is probably located in or overlaps amino-acids 25 to 42, but additional regions (1, 3, 4 and 5) are also important for the activation process, although not essential for synergy.

Repression, by EB1, of R-dependent transcription, also requires region 2 in the 'activation' domain

The observation that unbound EB1 can repress, in a dose dependent manner, the R-dependent transcription (Figure 2C),

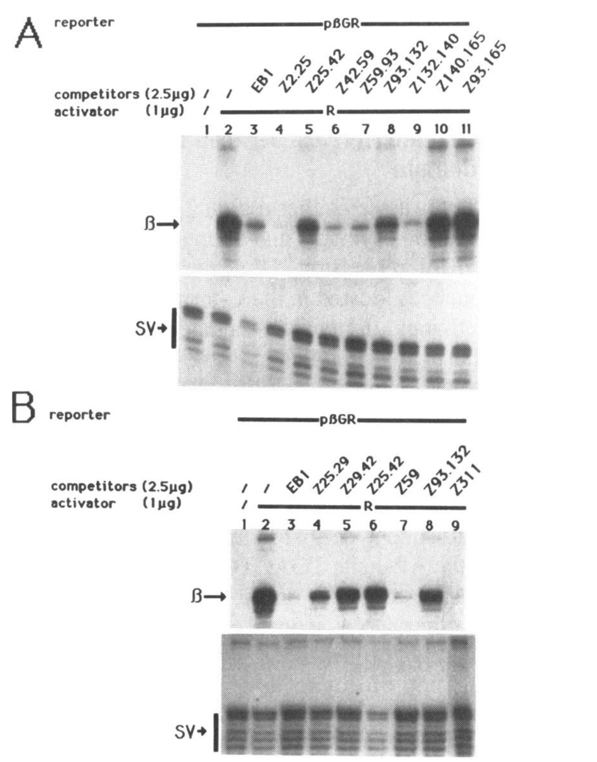

Figure 5. Repression of R-dependent activation requires region 2 and 5 in the EB1 activation domain. **A and B)** S1 nuclease mapping of RNAs extracted from HeLa cells transfected with the reporter gene pβGR alone (lanes 1), or together with EB1 or with EB1 variants as competitors. The genetic content of the EB1 variants presented in the upper part of panels **A** and **B**, is shown in Figure 3. SV denotes SV40 specifically initiated RNAs expressed from plasmid pSV2β transfected as an internal control. β represents the β-globin specific transcripts.

suggests that EB1 interacts with a factor(s) in a non productive manner. We were interested in determining which region(s) of EB1 is essential for its inhibitory effect on R. We therefore examined the repression capacity of the different N-terminal EB1 mutants localized in the nucleus. R-dependent activation of promoter pβGR was repressed by wild type EB1 (Figure 5A, lane 3). Deletion of region 2 (amino-acids 2 to 25), strongly impaired the repression potential of EB1 (Figure 5A, lane 5). Among the other mutated nuclear proteins examined, only deletion of region 5 (amino-acids 93 to 132), altered the repression competence of EB1 (Figure 5A, lane 8). However, deletion of regions 1, 3 and 4 (Figure 5A, lanes 4, 6 and 7), did not modify the repression competence of EB1. As expected, mutants not localized in the nuclei (Figure 5A, lanes 10 and 11) had no effect on the R-dependent activation.

Additional mutants were examined in a second series of experiment (Figure 5B), in which we included for comparative purpose, EB1 and mutants Z25.42 and Z93.132, already examined in Figure 5A. In mutants Z25.29 and Z29.42, sequencial deletions in region 2, progressively decreased the repression competence of EB1 (Figure 5B, lanes 4 and 5). Insertion of amino-acids EDLP at position 59 (Figure 5B, lane 7) had no effect on the repression competence of EB1. As already shown, deletion of amino-acids 93 to 132 (region 5), impaired the repression effect (lane 8). Finally, mutant Z311 had a single conservative amino-acid change in the alanine spacer, did not bind detectably to DNA (not shown), but was localized in the nucleus and could still repress (Figure 5B, lane 9). This last observation suggests that repression does not require a functional DNA binding domain in the EB1 protein. In conclusion, the results presented above imply that region 2 and region 5 contact directly or indirectly a factor(s) necessary for R to activate specific transcription.

Transcriptional interference between EB1 and R requires the EB1 basic domain

As shown above, it seems that region 2 (Figure 3A) is essential for both activation, synergy and repression, while region 5 is only essential for repression. However, it cannot be ruled out that EB1 sequences located in the C-terminus are also important for these activities. Extensive mutagenesis in this region is difficult to perform without impairing nuclear localization, or dimerization, and alter the stability of the protein variants (not shown). To study the role of sequences within the C-terminus of EB1, we constructed EB1 mutants carrying the Jun basic region A and B (mutant ZJ), the Jun basic region A (mutant ZJA), or the Jun alanine spacer plus region B (mutant ZJB)(Figure 3C). Since, activation and synergy are tested on promoter pM, where EB1 binds to the AP-1 site TGACTCA, we examined the binding of EB1, ZJ, ZJA and ZJB on the AP-1 site (probe Pmo), as compared to other DNA sequences where EB1 DNA-binding ability is different (probes DR1, DR2, DR3 and DR4).

First the binding of in vitro expressed EB1 and ZJ proteins, was performed at 20°C or 4°C (Figure 6A). When binding was done at 20°C, EB1 bound to the four DNA probes Pmo, DR1, DR2 and DR4 with different affinities (Figure 6B, lanes 1 to 6) but binding of ZJ to the five DNA probes was almost undetectable (Figure 6B, lanes 7 to 11). At 4°C, EB1 bound to probes Pmo, DR1, DR2, and DR4 with almost equal affinities (Figure 6B, lanes 13, 14, 15 and 17), very poorly to probe DR3 (lane 16), and ZJ bound only to probe Pmo (Figure 6B, lane 18). In summary, the Jun basic region in EB1 allowed mutant ZJ to bind only to the AP-1 site TGACTCA and the binding was detectable only at 4°C.

To further define which regions of the Jun and EB1 basic domains carry the DNA binding specificity, we compared EB1, ZJ, ZJA and ZJB variants for their ability to bind in vitro, either at 20°C or at 4°C, to the AP-1 site TGACTCA and to the specific

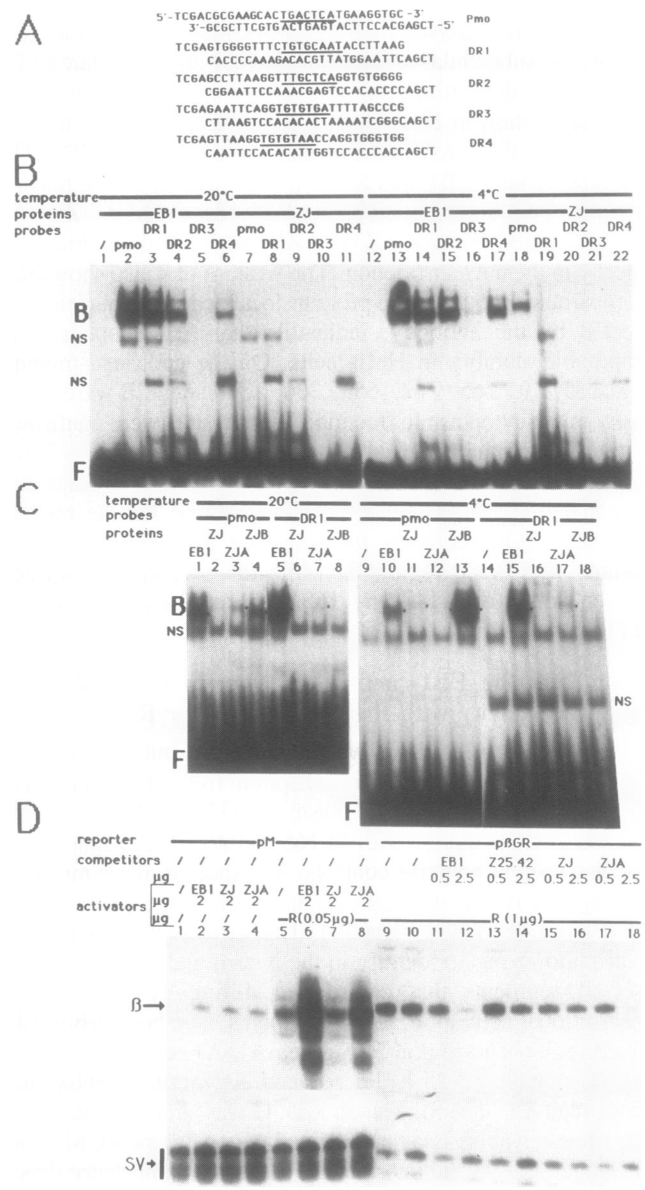

Figure 6. DNA binding specificity and activation-repression potential of jun-EB1 hybrid proteins. **A)** Schematic representation of DNA probes used for mobility shift assays. The EB1 binding sites are underlined. Probe Pmo contains the AP-1 site TGACTCA. All the EB1 binding sites were synthesized with their natural flanking sequences. **B and C)** Mobility shift assays were performed either at 20°C or at 4°C, with the proteins and the probes described at the top of the panels. In C, the specific complexes were labelled with black squares. F, represents free probe, B represents protein-bound probe and NS represents non-specific complexes. **D)** S1 nuclease analysis (β) of RNAs extracted from HeLa cells transfected with the reporter gene pM (lanes 1 to 8), or with the reporter gene pβGR (lanes 9 to 18). Reporter gene pM was transfected alone (lane 1), or in the presence of EB1 (lane 2), or together with the EB1 mutants presented in the upper part of the panel (lanes 3 and 4), or with R (lane 5), or with R plus EB1 (lane 6), or with R plus EB1 mutant proteins (lanes 7 and 8). The reporter gene pβGR was transfected with an R-expression vector (lanes 9 and 10), or with an R-expression vector plus two concentrations of competitor protein-expression vectors as presented in the upper part of the pannel. S1 nuclease analysis of RNAs expressed from plasmid pSV2β cotransfected as an internal control is also presented (SV).

EB1 site DR1 (Figure 6C). As vizualized by mobility shift assay, complex formation on the AP-1 site was detected at 20°C with EB1, ZJA and ZJB (Figure 6C, lanes 1, 3 and 4), but not with ZJ (lane 2). Complex formation was also detected at 20°C on the EB1 site DR1, but only with EB1 and ZJA (Figure 6C, lanes 5 and 7). The same assays performed at 4°C show that complex formation was detected on the AP-1 site with proteins EB1, ZJ, ZJA and ZJB (Figure 6C, lanes 10, 11, 12 and 13). Finally complex formation on the EB1 site DR1 was only detected at 4°C with EB1 and ZJA (Figure 6C, lanes 15 and 17). In conclusion, the Jun basic region A in EB1 allowed mutant ZJA to bind to the AP-1 site and the DR1 site at both 20 and 4°C, as does EB1, but with a reduced affinity as compared to EB1. However, mutant ZJB bound only to the AP-1 site, and the binding was about ten times more efficient at 4°C than at 20°C, as with mutant ZJ, or with the Jun homodimers (not shown).

Although EB1 and the nuclear variants ZJ and ZJA did not bind with the same affinity to the AP-1 site, we nevertheless investigated their activation capacity on promoter PM. As shown in Figure 6D, mutant ZJ (lane 3) and ZJA (lane 4) increased the basal activity of promoter PM, similarly to EB1 (lane 2). As compared to the EB1-induced activation (lane 2), and the R-induced activation (lane 5), coexpression of EB1 and R (lane 6) or of ZJA and R (lane 8) had a more than multiplicative effect. Conversely, coexpression of ZJ and R (lane 7), had an effect similar to R alone (lane 5). In conclusion, synergism was observed between EB1 and R, or ZJA and R, but not between ZJ and R. This could be explained by the reduced affinity of ZJ for the AP-1 site. However, the three proteins EB1, ZJ and ZJA should be able to repress efficiently the R-dependent activation, since they all carry region 2 and region 5 in the activation domain, and since repression does not require a functional DNA-binding domain (mutant Z311, Figure 5B, lane 9). As shown in figure 6D, the R-induced activation of promoter pβGR (lanes 9 and 10), was efficiently repressed by EB1 (lanes 11 and 12). As expected, a mutant lacking region 2 in the activation domain did not repress efficiently (lanes 13 and 14). Surprisingly, mutant ZJ did not repress (lanes 15 and 16), although mutant ZJA repressed like EB1 (lanes 17 and 18). In conclusion, our results suggest that the basic region, and probably basic region B, is required for EB1 to repress the R-dependent activation of transcription. Again, the level of SV40 specific transcription was not affected at the two concentrations of competitor used (Figure 6D, lanes 9 to 18, lower panel), indicating that the factor(s) sequestered by EB1 is not essential for SV40 early transcription.

DISCUSSION

Our results demonstrate that EB1 can act synergistically with the EBV sequence-specific DNA binding enhancer factor R, and that unbound EB1 represses the R-induced activation by a mechanism related to 'squelching' (31). Repression and synergy, require both region 2 in the 'activation' domain and the basic region of EB1, which is also required for nuclear localization and stable specific binding to DNA.

The EB1 basic domain B directs the protein to the nucleus, and mediates specific and stable interaction with DNA

It has been proposed that DNA binding specificities of GCN4 and C/EBP correlate with the basic region (BR-A, alanine spacer, BR-B) (32). We show here that binding specificities of EB1-Jun hybrid proteins also correlate with the basic region, since exchange of the EB1 basic region for the homologous regions of Jun modified the binding specificity of EB1 to that of Jun. Moreover, the low DNA-binding ability of Jun homodimers for the AP-1 site, as compared to the high DNA-binding ability of Jun/Fos heterodimers, is thought to reflect differences in the ability of their leucine zippers to form dimers (33). However, our results show that the low DNA-binding ability of the EB1-Jun hybrid ZJ for the AP-1 site is seen when the basic region of Jun is located next to a dimerization domain that otherwise allows stable binding of EB1 to the AP-1 site (Figure 5C, lane 2). We also observed that ZJ and EB1 could form stable heterodimers at temperatures over 20°C (not shown). These results suggest that low DNA-binding ability is also an intrinsic property of the jun basic region, and probably basic region B.

The basic region B of EB1, contains also a signal required for nuclear localization with the sequence RKCRAKFK (nls2: nuclear localization signal 2). Single or multiple amino-acid substitutions in this sequence restricted mutants Z306, Z310 to the cytoplasm, but also impaired their binding to DNA in vitro (not shown). Therefore mutants Z306 and Z310 might be transported to the nucleus but will not stay into the nucleus because they do not bind to DNA (Figure 3). However, this is unlikely since mutant Z311 does not bind to DNA (not shown), but is nuclear (Figure 3). Moreover, although mutant ZJB binds to DNA in vitro, the Jun basic region B in mutant ZJB cannot compensate for the loss nls2, since ZJB is found in the cytoplasm. They also suggest that Jun basic region A contains a peptide that can compensate for the loss of nls2. There is a second signal required for the nuclear localization of EB1 located between amino-acids 140 and 165. In this region, one sequence rich in basic amino-acids, RRTRKP (nls1), is related to the short amino-acid sequences found in other viral nuclear proteins (34). At present we do not know the function of nls1 and nls2. It could be that one signal is required for transport to the nuclei while the other is required for remaining in the nucleus (35). Our results have therefore assigned several novel important functions, i.e., nuclear localization, stability of DNA-binding and specificity of DNA-interaction, to a protein domain which was only described as required for specific interaction with cognate DNA sequences, the basic region B.

Activation and synergy

Our deletional mutagenesis studies indicates that the EB1 domain localized between amino-acids 2 and 132, contributes to efficient activation of specific initiation of transcription. In this domain, one segment called region 2, is essential for the activation process, while other segments called 1, 3, 4 and 5 decreased the activation capacity of EB1 to different extents. These regions do not play any role in the capacity of EB1 to interact specifically as a dimer to DNA (not shown). By these properties, it seems that the N-terminal region of EB1 is similar to 'activating' domains identified in other transcription factors (36; 37; 38; 39; 40). However, it remains to be established if region 1 to region 5 maintain their activating properties when linked individually to an heterologous DNA-binding domain.

The domain required for transcriptional activation has no clear structural features related to transactivation domains of other proteins. It is not enriched in acidic amino-acids (36; 37). However, regions 3, 4 and 5 have a higher content in prolines than region 1 and region 2 (38; 39), and region 4 and region 5 are rich in glutamines (40). Moreover, none of these regions seems to be able to adopt an ordered protein structure.

Region 2, is essential for EB1-activation of transcription and for synergy between EB1 and R. However, exchanging the EB1 basic region for the one of Jun (mutant ZJ, Figure 3C), did not modify the extent of specific initiation of transcription by ZJ as compared to EB1 (Figure 6D, lanes 2 and 3), whereas it impaired synergy between EB1 and ZJ (Figure 6D, lanes 7). These results suggest that synergy requires functional interactions between EB1 and R, mediated by both the basic region and the 'activating' region 2. These interactions have been indirectly vizualised by transcriptional interference/squelching experiments.

Transcriptional interference

Our results demonstrate that unbound EB1 can function as a potent inhibitor of the R-activated transcription, and this inhibition is seen either in the absence of an EB1 binding site on the reporter gene, or in the absence of a functional DNA-binding domain in the protein (mutant Z311, figure 5B). This inhibitory effect looks like 'squelching' experiments, in which overexpressed unbound activators, sequester cellular auxillary factors required by activators to contact the basal transcription apparatus, resulting in repression of activator-induced transcription (31; 41; 42; 43; 44; 45). Repression by EB1 is also impaired when region 2 is deleted, but surprisingly, it is also impaired by deletion of region 5 and by exchanging the EB1 basic region for the Jun basic region (mutant ZJ). These results indicate that both EB1's region 2 and basic region contact a factor(s) essential for R-dependent activation, and probably for synergy. The fact that region 5 is dispensable for synergy, but necessary for repression, reflects probably differences in the conformations of bound or unbound EB1 involved in the interaction between EB1 and the factor(s). However, we also observed that unbound R cannot repress EB1-dependent activation of transcription. This data suggests that EB1 and R do not directly contact the same factors. This is not surprising, since it has been reported that distinct classes of transcriptional activating domains may interact with different cellular targets, resulting in non-reciprocal 'squelching' (42; 45). Therefore, the lack of repression of EB1-activation by R, probably reflects fondamental structural differences between the activation domains of EB1 and R.

In conclusion, our studies suggest that several domains in the EB1 protein are involved in direct interactions with a factor required for R-dependent activation of transcription and for synergy between EB1 and R. Understanding of the complex interactions between EB1 and R awaits purified factors and in vitro reconstituted systems.

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